

UNCLASSIFIED

AD 410502

DEFENSE DOCUMENTATION CENTER

FOR

SCIENTIFIC AND TECHNICAL INFORMATION

CAMERON STATION, ALEXANDRIA, VIRGINIA



UNCLASSIFIED

NOTICE: When government or other drawings, specifications or other data are used for any purpose other than in connection with a definitely related government procurement operation, the U. S. Government thereby incurs no responsibility, nor any obligation whatsoever; and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use or sell any patented invention that may in any way be related thereto.

41052



SIXTH US ARMY
MEDICAL LABORATORY
FORT BAKER
CALIFORNIA



RESEARCH REPORT

ANNUAL RESEARCH PROGRESS REPORT
REPORTS CONTROL SYMBOL MEDDH-288
30 JUNE 1963

PROJECT NO. 6X61-01-001-01

VOLUME II

SIXTH US ARMY MEDICAL LABORATORY

Fort Baker, California

ANNUAL RESEARCH REPORT

Volume 2.

Reports Control Symbol MEDDH-288

Qualified requestors may obtain copies of this report from ASTIA

FINAL REPORT

Project 6X61-01-001-01, Communicable Diseases

Task 1, Enhancement of Specificity of the Fluorescent Treponemal
Antibody Test as Compared to the TPI Test

Reporting Installation: Sixth US Army Medical Laboratory
Fort Baker, California

Serology Section
Division of Immunology

Period Covered by Report: 1 Jul 1962 through 30 June 1963

Principal Investigators: Albert Leibovitz, Lt Col, MSC
Thomas R. Oberhofer, 1st Lt, MSC
Theodore N. Diestelhorst, B.S.
John T. Meacham, Jr., PFC

Reports Control Symbol: MEDDH-288

Security Classification UNCLASSIFIED

ABSTRACT

Project 6X61-01-001-01 Title: Communicable Diseases

Task No. 1 Title: Enhancement of Specificity of the Fluorescent Treponemal Antibody Test as Compared to the TPI Test

Reporting Installation: Sixth US Army Medical Laboratory
Fort Baker, California

Period Covered by Report: 1 Jul 1962 through 30 June 1963

Authors: Albert Leibovitz, Lt Col, MSC
Thomas R. Oberhofer, 1st Lt, MSC
Theodore N. Diestelhorst, B.S.
John T. Meacham, Jr., PFC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

The material presented herein is the final report on the comparative studies of the TPI and FTA tests. Modifications in the FTA test were introduced to insure uniformity and to eliminate persistent stumbling blocks in the test. Critical points of investigation were the preparation of treponemal antigen, fixation of the antigen to glass slides and standardization of the test procedure. A total of 927 serum specimens were examined. The first 283 specimens were processed employing the FTA 100 and FTA 200 tests and the remainder by the FTA 100 test only. The FTA 200 test was 89 per cent in agreement with the TPI test and the FTA 100 test approximately 91 per cent.

The use of the FTA test in the diagnosis of syphilis has realized distinct advantages over the TPI test, which are: (1) maintenance of motile treponemes is not required, (2) ease in the preparation of antigen, (3) ease and rapidity in performance of the test procedure, (4) relatively little expense involved following initial costs, (5) availability of commercial reagents, and (6) highly trained technicians are not required to perform the test.

The findings of these comparative studies have been submitted to and accepted for publication by The American Journal of Clinical Pathology.

BODY OF REPORT

Project No. 6X61-01-001-01

Title: Communicable Diseases

Task No. 1

Title: Enhancement of Specificity of the Fluorescent Treponemal Antibody Test as Compared to the TPI Test

Description:

Since Deacon and associates (4) advocated the Fluorescent Treponemal Antibody (FTA) Test for syphilis in 1957, efforts have been made by the above authors (5-7) and others (3,8,10) to enhance the specificity of this test. Establishment of the FTA test as a standard diagnostic aid to rule out Biological False Positive (BFP) reactors would enable many serology laboratories to perform this test rather than transmitting specimens to one of the relatively few laboratories established to perform the Treponema Pallidum Immobilization (TPI) Test. Although all reagents for the FTA test are available commercially, difficulty has been encountered in the production of an antigen that readily adheres to glass slides (3,10). This problem has been further resolved and the specificity of the FTA test as correlated to the TPI test is enhanced by moderate modifications of the techniques reported by various authors (3-5,8).

Progress:

MATERIALS AND METHODS

TPI test procedure: The sustaining medium was prepared by the technique of Boak and Miller (1). Blood aseptically removed from healthy, adult male rabbits by cardiac puncture was centrifuged and the serum removed. The clear serum not used immediately was stored at -20C for no longer than one week. The serum for use was inactivated for 45 min. at 60C and then diluted with equal parts 0.9 per cent saline to compose the basal medium. Only serum yielding a non-reactive Cardiolipin microflocculation was used in the sustaining medium.

Selected rabbits for antigen passage were administered cortisone acetate after the technique of Turner and Hollander (16). The rabbits received 0.7 ml. (25 mg./ml.) subcutaneously 24 hours prior to inoculation of treponemes, again after inoculation and then daily until sacrificed.

Nichols virulent strain of Treponema pallidum was used for antigen production. Freshly harvested treponemes were adjusted to 20-30 organisms per high-powered field (HPF) in basal medium and 1 ml. was injected into each testicle using a 5 ml. Luer-Lock syringe and

and 20-gauge needle. These animals were individually caged in an air-conditioned room stabilized at 15C. The use of cortisone permitted a maximum yield of treponemes over a 9- to 12-day incubation period, thus permitting planned harvest days. Cortisoned rabbits rarely developed a demonstrable orchitis during the prescribed incubation period. However, they were palpated to ascertain possible orchitis formation before sacrificing. When such occurred, the testes usually contained few treponemes. These lots were discarded unless they were the only remaining source of maintaining the strain. The TPI test procedure of Nelson and Mayer (13) was performed on all serum specimens.

FTA test procedure:

Antigen: The Nichols strain of T. pallidum as cultivated in the rabbit testicle for the TPI test also served as antigen for the FTA test. Following extraction from the rabbit tissue in basal medium, the treponemal suspension was centrifuged for 10 min. at 600g to remove gross cell debris. The supernate was transferred to a 15 x 75 mm. plastic Spinco centrifuge tube¹ containing an equal volume of 0.002 per cent sodium hypochlorite (NaClO) in 0.9 per cent NaCl solution (3,10), mixed well and spun at 4000g for 10 min. The supernate was discarded and the organisms were resuspended in fresh NaClO normal saline solution and re-centrifuged. Following decanting, the treponemes were suspended in a 5 per cent dimethyl sulfoxide² - 10 per cent normal rabbit serum solution prepared in 0.9 per cent saline to yield a final count of 20 to 30 organisms per HPF. The antigen suspension was divided into 0.5 ml. aliquots in small, screw-cap glass vials and frozen at -65C. Once thawed for use, the antigen suspension was stored at 4C and not refrozen.

Conjugate: Commercially prepared fluorescein goat anti-human globulin³ was used throughout the test. Each lot of conjugate was titered and optimal dilutions (usually 1:20 to 1:40) were prepared in 0.01M phosphate buffered saline (PBS), pH 7.2. The diluted conjugate was divided into convenient aliquots and frozen at -20C.

Patients' sera: Serum specimens for routine TPI tests were submitted to this laboratory from the western half of the United States, including Alaska and Hawaii. In most instances, the patients had positive STS tests without definitive clinical evidence of syphilis. However, case histories were usually scanty and the underlying cause of the positive STS test could not be readily determined. Therefore, no attempt was made to categorize sera as from probable syphilitic

- 1 Spinco Division, Beckman Instrument Company, Palo Alto, California
- 2 J. T. Baker Chemical Company, Phillipsburg, New Jersey
- 3 Baltimore Biological Laboratories, Inc., Baltimore 18, Maryland

or BFP reactors. Regardless of TPI findings, a second specimen was requested to confirm the results. When results of the second test were at variance with the first, additional specimens were requested. Sera were stored at -20C and were inactivated at 56C for 30 min. prior to testing.

Standardized test procedure: A total of 927 serum specimens were examined in this study. The first 283 specimens were processed after the technique of Fife and associates (8) for both the FTA 100 (8) and FTA 200 (5) tests. The remainder were processed by the technique evolved in this laboratory for the FTA 100 test. Special slides with two pre-etched circles about 1 cm. in diameter and etched end for labeling were obtained from Aloe Scientific Co.¹ These were placed in 95 per cent ethanol overnight and wiped dry with gauze.

1. Using a disposable pipette, a minimal amount of antigen (less than 0.01 ml.) containing 20 to 30 organisms per HPF was placed on each of the two circles and spread with a small, footed glass rod to cover the entire circle (excess antigen resulted in wrinkling on drying and masking of the organisms) and air dried. 2. The dried slides were fixed in a precooled (-78C) acetone-dry ice bath for 2 to 3 min. Fixing in the dry ice bath from 2 to 10 min. gave comparable results. The slides were removed from the bath, drained and air dried. 3. Inactivated patient's serum (about 0.03 ml.) diluted 1:100 in PBS was placed over each smear and spread to cover the entire area enclosed by the etched circle. 4. The slides were placed on a level tray, covered with aluminum foil to prevent drying, and incubated at 34C for 30 min. 5. Following incubation, the excess sera was removed by rinsing with PBS and then the slides were gently blotted. 6. Optimally diluted fluorescein conjugate (about 0.03 ml.) was placed over each smear and spread to cover the entire circled area and step 4 was repeated. 7. Excess conjugate was removed by rinsing in PBS and then immersing for 5 min. each in 2 fresh PBS baths and the slides air dried. 8. Each smear was mounted with buffered glycerol (9 parts glycerol plus 1 part PBS) and coverslips added. The slides were read immediately or stored overnight at 4C. Controls were reactive serums yielding maximum fluorescence, minimum fluorescence, non-reactive serum and an antigen control treated only with conjugate. Interpretation of test serums were as follows: serums yielding maximum fluorescence (4+) to moderate fluorescence (2+) were designated as reactive; serums yielding minimum fluorescence (1+) or rendering the organisms barely visible (2) or - were designated as non-reactive (5).

Fluorescent microscope: A Zeiss fluorescent microscope which allowed visualization of treponemes by regular darkfield before

¹ Aloe Scientific Company, St. Louis, Missouri

applying ultraviolet light was used throughout this study. Optimal fluorescence of the treponemes were obtained using a UG 2 exciter filter combined with barrier filters that excluded all wave lengths under 500 A°.

RESULTS

The findings on 927 consecutive serum specimens submitted to this laboratory for routine TPI tests form the basis for this report. The initial 283 specimens served to compare the FTA 100, FTA 200 and the TPI tests after the technique of Fife and co-workers (8). Many of the FTA slides had to be repeated due to excessive loss of treponemes during processing. This loss necessitates slide preparation with initial concentrations from 50 to 100 treponemes per HPF to retain sufficient organisms for diagnostic purposes. When organisms were retained, however, excellent fluorescence was obtained on positive slides. The FTA 100 test correlated closer with the TPI findings and this test was used for the remainder of the study (Tables 1, 2, and 3).

TABLE 1

Comparison of the TPI, FTA 100 and FTA 200 Tests on 927 Serum Specimens Routinely Submitted for TPI Studies

Number Sera	R*	TPI TEST				FTA 100		FTA 200	
		WR	NR	I	AC	R	NR	R	NR
283	31	13	118	20	1	166	117	151	132
<u>644</u>	<u>276</u>	<u>13</u>	<u>214</u>	<u>133</u>	<u>8</u>	<u>363</u>	<u>281</u>	-	-
927	407	26	332	153	9	529	398		

* R: Reactive, WR: Weakly Reactive, NR: Non-reactive

I: Invalid, AC: Anticomplementary

TABLE 2

Comparison of the TPI, FTA 100 and FTA 200 Tests in Which Invalid
and Anticomplementary TPI Sera are Eliminated from the Study
(765 Specimens)

Number Sera	TPI		FTA 100		FTA 200	
	R*	NR**	R	NR	R	NR
262	144 (55)***	118 (45)	148 (56)	114 (44)	137 (52)	125 (48)
<u>503</u>	<u>289</u> (57)	<u>214</u> (43)	<u>282</u> (56)	<u>221</u> (44)	-	-
765	433 (57)	332 (43)	430 (56)	335 (44)		

* R: Reactive (includes both R and WR TPI findings)

** NR: Non-Reactive

*** Parenthesis indicate percentages

Although treponemes can be stored at 4C for several weeks and still retain their antigenic structure for the FTA t4st, it was noted that they gradually became thready and a marked diminution in fluorescence occurred. As the FTA test performed on freshly harvested treponemes invariably yielded brilliant fluorescence, attempts were made to preserve large lots of antigen under conditions that minimized loss of capsular material. Deep freezing of the organisms or lyophilization yielded usable organisms, but again with a diminution of fluorescence. Glycerol (9) could not be used to preserve the capsule as upon drying of the suspension for staining this chemical would mask the organisms. Dimethyl sulfoxide has been used successfully in the preservation of primary tissue cell lines in the deep freeze (14) and it was noted that from 2.5 to 10 per cent dimethyl sulfoxide in the presence of 10 to 50 per cent inactivated normal rabbit serum allowed the deep freezing of treponemes over at least a 6-month period and still yield brilliantly fluorescing organisms.

TABLE 3

Comparison of Agreement of 765 FTA and TPI Results in Which Invalid
and Anticomplementary Results are Eliminated

	No. Reactive in Nonreactive Sera Both Tests*		FTA-Reactive TPI-Nonreactive		FTA-Nonreactive TPI-Reactive	
FTA 200	262	125 (48%)	107	(41%)	11	(4%)
FTA 100	765	396 (51.8%)	297	(38.8%)	36	(4.7%)

* Weakly reactive TPI results included with reactive

The findings of Covert and co-workers (3) and Kent and associates (10) that 0.002 per cent sodium hypochlorite in normal saline enhanced adherence of the treponemes to glass slides was confirmed. However, it was noted that in the absence of serum, there was a marked diminution in fluorescence. Addition of serum enhanced fluorescence but reduced adherence. Addition of dimethyl sulfoxide, however, increased adherence in the presence of serum. Graded experiments indicated that the use of 5 per cent dimethyl sulfoxide plus 10 per cent inactivated normal rabbit serum in normal saline consistently yielded brilliant fluorescence upon processing with but slight loss of treponemes. This method of antigen preparation was employed on the remaining 644 specimens tested by the FTA 100 technique and compared to the TPI test. Storage of organisms in convenient aliquots allowed efficient utilization of the antigen. Once thawed, the organisms were not refrozen but stored at 4C and were usable for at least 2 weeks.

Of the 927 specimens tested, the TPI test was valid in only 765 cases; 153 specimens yielded invalid results and 9 were anticomplementary. To compare the FTA tests to the TPI test, both the weakly reactive and the reactive TPI were considered as reactive. These results are summarized in Table 3. There is approximately a 91 per cent correlation in exact findings with the two tests. These results agree closely with the findings of Fife and associates. (8).

Two or more specimens on the same patient were received from 225 patients. All specimens from 170 patients gave valid TPI findings and were comparable to the FTA test. Exact findings on all specimens on 143 of these patients (86 per cent) were obtained with both tests. Those tests in which exact findings were not obtained in all specimens are of interest in attempting to make a definitive diagnosis. A possible plan in evaluation is demonstrated in Table 4. The value of repeat serum specimens is evident.

Multiple specimens received on 55 patients in which one or more yielded invalid findings in the TPI test were evaluated by repeat specimens and the FTA test in attempting to make a specific diagnosis. Table 5 presents these findings and the value of the FTA test is again evident although additional specimens are needed in some of these cases before a definitive diagnosis can be made.

The weakly reactive (WR) TPI test was noted (2) to be the most difficult to obtain consistent findings on repeat examination of the same specimen. However, further demonstration of the close correlation of the FTA 100 test to the TPI test was found in those patients that showed a consistent WR TPI in repeat specimens. Nine patients came in this category and the results are tabulated in Table 6.

TABLE 4

Evaluation of Discrepancies in Comparison of the FTA 100 Test to the TPI Test When Multiple Specimens are Received on the Same Patient

EXAMPLE	TPI SPECIMENS			FTA 100 SPECIMENS			OCCURRENCES	PROBABLE DIAGNOSIS
	1	2	3	1	2	3		
1	0	+		0	+		2	A**
2	0	0		0	+		4	A
3	+	+		0	+		4	B***
4	+	+	+	+	0	+	2	B
5	+	+	+	0	+	+	1	B
6	0	+	+	0	+	0	1	A
7	+	0		+	0		4	A
8	+	+		+	0		4	A or B
9	+	0		+	+		1	A or B
10	0	0		+	0		7	A
11	0	0		+	+		1	A
12	+	0	0	0	0	0	1	C***
13	0	+		+	0		1	A

* +: Includes weakly reactive (WR) and reactive (R) results

A: Inconclusive: another specimen desired

B: Probable positive for anti-treponemal antibody

C: Probable BFP

: Basis of Probable Diagnosis: <25% + = C
 25-50% + = A
 50-75% + = A or B
 over 75% + = B

TABLE 5

FTA 100 Test Findings with Invalid TPI Results:
 The Value of Repeat Specimens in Assisting in the Diagnosis

1	TPI TEST SPECIMENS			4	FTA 100 TEST SPECIMENS			4	OCCURRENCES
	1	2	3		1	2	3		
	a	b			+	+			6
	0 ^c	+			0	0			7
	+	0			0	0			4
	+	+			+	+			3
	+	+			0	0			2
	+	+			0	0			2
	+	+	+		+	+	+		6
	+	+	+		+	+	+		2
	+	+	+		0	0	0		1
	+	+	+		0	0	+		1
	0	+	0		0	+	0		1
	+	+	+	+	+	+	0	+	1
	0	+	+	0	+	0	0	+	1

a. +, Includes weakly reactive and reactive findings

b. +, Invalid

c. 0, Non-reactive

TABLE 6

Examination of repeat specimens from patients yielding a Weakly
Reactive (WR) TPI test.

FTA IGG SPECIMEN			TPI SPECIMEN			OCCURRENCE
1	2	3	1	2	3	
0 ^a	+		WR ^c	+		1
0	+		0	WR		1
+	+		WR	WR		3
+	+		WR	+		1
+	0		WR	0		1
+	+	+	WR	WR	WR	1
0	0	0	WR	0	0	1

a. 0 = non-reactive

b. + = reactive

c. WR = weakly reactive

DISCUSSION

In the original paper by Deacon and associates (4) on the FTA test, two methods of processing treponemal smears are presented. Although method "A" correlated more closely with their TPI findings, method "B" was chosen for further studies as earlier antibody formation, as indicated by fluorescence, was detected by this method in the disease of treponemal infected rabbits. Montgomery and co-workers (12) also used method "B" and found the FTA test to correlate with reagin sensitivity. In our efforts to prevent the loss of treponemes during processing, the rotation of slides during incubation with patient's serum and with conjugated serum was eliminated (method "A"). In addition, the 10 min. soak following incubation with patient's serum was eliminated. Comparison of findings of the first 283 specimens that were processed after the technique of Fife and associates (8), in which both rotation and strenuous soaking is advocated, with the remaining 644 specimens in which these steps are eliminated, indicate that almost similar sensitivity and specificity are obtained (Tables 1, 2 and 3).

The finding that inactivated normal rabbit serum would enhance fluorescence of treponemes resulted from the attempt to preserve antigen over an extended period in the deep freeze. This may have been due to preservation of the treponemal capsule. However, the greater the concentration of serum employed, the more readily the treponemes were lost during processing. Sodium hypochlorite or NaClO plus dimethyl sulfoxide, without serum, enhanced the adherence of the treponemes to the slides, but the degree of fluorescence was markedly diminished when organisms were stored at 4C for several weeks, or lyophilized, or frozen and thawed, as compared to freshly harvested treponemes. The ratio of 5 per cent dimethyl sulfoxide to 10 per cent rabbit serum in normal saline was found to be the best combination to assure adherence and brilliant fluorescence. The use of 20 to 30 treponemes per HPF yielded consistently good slides with adequate numbers of organisms for easy determination of the degree of fluorescence. The maintenance of deep frozen treponemes in this suspension allows the preparation of large lots of antigen and assurance of similar findings for the duration of its use. Once an aliquot is thawed, it should be used within 14 days or discarded.

Indirect evidence indicates that the fluorescent antibody may differ from the TPI antibody in that the former is most pronounced when the capsule is still intact. Metzger and associates (11) have demonstrated that the TPI reaction rate can be increased when the capsule is dissolved with lysozyme. However, the close correlation in specificity and sensitivity between the FTA 100 and the TPI tests as demonstrated in this study and by others (5,6,8) indicates a close relationship, if not the same antibody. Further study on this correlation is indicated.

Covert and associates (3) and Kent and co-workers (10) only obtained an 80 per cent correlation between the FTA and the TPI tests. However, they were using a 40 hr. TPI test and a 1:500 dilution of their conjugate for the FTA test. They also advocate the use of the Reiter treponeme for the FTA test. Deacon and Hunter (7) indicate that use of the Reiter treponeme may lead to fallacious findings as this organism is related to T. pallidum only through a "non-specific" antigen that can also be found in the mouth treponemes and in T. zuelzeri, a saprophytic mud organism.

Reliance on a single TPI or FTA test for the diagnosis of syphilis can be misleading in at least 15 per cent of findings. Paired specimens from patients to confirm the initial FTA and TPI findings enhances the reliability and value of these tests.

The present study also illustrates the practicability and value of the FTA 100 test as a diagnostic test when considering the large percentage of invalid results obtained with the TPI test. Of the 927 serum specimens examined in this study, 162 yielded invalid or anticomplementary results in the TPI test. Although this problem is partially resolved through use of the euglobulin precipitation procedure of Sanders and associates (15), usable results were obtained in all instances using the FTA technique.

SUMMARY

1. The production of Treponema pallidum antigen that readily adheres to glass slides is further resolved through suspension of NaClO treated organisms in normal saline containing 5 per cent dimethyl sulfoxide and 10 per cent inactivated normal rabbit serum.
2. This solution also permits the storage of treponemes in the deep freeze without diminution of fluorescence.
3. The FTA 100 test was found to be as specific and as sensitive as the TPI test to rule out BFP reactors.
4. Paired serum specimens enhanced the reliability of both the FTA and TPI tests in rendering a definitive diagnosis.

BIBLIOGRAPHY

1. BOAK, R. A., AND MILLER, J. N.: A simple medium for maintaining the viability of Treponema pallidum in the treponema pallidum immobilization test. Am. J. Syph., 38: 429-433, 1954.
2. BROWNE, A. S., BISSETT, M. L., AND MICHELbacher, M. M.: Reproducibility of TPI and TPCF tests. Am. J. Clin. Path., 31: 205-212, 1959.
3. COVERT, S. V., KENT, J. F., AND STEVENS, R. W.: Fluorescent treponemal antibody test using the Reiter treponema. Proc. Soc. Exper. Biol. & Med., 106: 729-731, 1961.
4. DEACON, W. E., FALCONE, V. H., AND HARRIS, A.: A fluorescent test for treponemal antibodies. Proc. Soc. Exper. Biol. & Med., 96: 477-480, 1957.
5. DEACON, W. E., FREEMAN, E. M., AND HARRIS, A.: Fluorescent treponemal antibody test. Modification based on quantitation (FTA 200). Proc. Soc. Exper. Biol. & Med., 103: 827-829, 1960.
6. DEACON, W. E., AND FREEMAN, E. M.: Fluorescent treponemal antibody studies. J. Invest. Dermat., 34: 249-253, 1960.
7. DEACON, W. E., AND HUNTER, E. F.: Treponemal antigens as related to identification and syphilis serology. Proc. Soc. Exper. Biol. & Med., 110: 352-356, 1962.
8. FIFE, E. H., JR., BRYAN, B. M., SANDERS, R. W., AND MUSCHEL, L. H.: Evaluation of the fluorescent treponemal antibody (FTA) test for syphilis. Comparison with the Treponema pallidum immobilization (TPI) test. Am. J. Clin. Path., 36: 105-113, 1961.
9. HOLLANDER, D. H., AND NELL, E. E.: Improved preservation of Treponema pallidum and other bacteria by freezing with glycerol. Appl. Microbiol., 2: 164-170, 1954.
10. KENT, J. F., COVERT, S. V., REILLY, H. W., KINCH, W. H., AND LAWSON, W. B.: Evaluation of fluorescent treponemal antibody (RFTA and FTA II) and other tests (RPCF and TPI) for syphilis. Proc. Soc. Exper. Biol. & Med., 109: 584-589, 1962.
11. METZGER, M., HARDY, P. H., JR., AND NELL, E. E.: Influence of lysozyme upon the treponeme immobilization reaction. Am. J. Hyg., 73: 236-244, 1961.
12. MONTGOMERY, G. H., SUHRland, S., AND KNOX, J. M.: Observations concerning fluorescent treponemal antibody test for syphilis. J. Invest. Dermat., 35: 95-101, 1960.

13. NELSON, R. A., JR., AND MAYER, M. M.: Immobilization of Treponema pallidum in vitro by antibody produced in syphilitic infection. J. Exper. Med., 89: 369-393, 1949.
14. PORTER FIELD, J. S., AND ASHWOOD-SMITH, M. J.: Preservation of cells in tissue culture by glycerol and dimethyl sulfoxide. Nature, London, 193: 548-552, 1962.
15. SANDERS, R. W., PIPER, D. R., HOOK, W. A., AND MUSCHEL, L. H.: Non-specific immobilization in the Treponema pallidum Immobilization (TPI) test. Am. J. Clin. Path., 33: 135-137, 1960.
16. TURNER, T. B., AND HOLLANDER, D. H.: Biology of the treponematoses. Monograph Series No. 35. World Health Organization, Palais des Nations, Geneva, 84-88, 1957.

ANNUAL PROGRESS REPORT

Project 6X61-01-001-01, Preventive Medicine - Communicable Diseases

Task 2, Studies on Penicillin Treatment Failures of Gonorrhea

Reporting Installations: Sixth US Army Medical Laboratory
Fort Baker, California

US Army Hospital
Fort Huachuca, Arizona

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Adrian D. Mandel, Major, MSC
Edward Kopplin, Jr., Capt, MSC*
Robert S. Croissant, Capt, MC*
George J. Omi, B.S.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

*U. S. Army Hospital, Fort Huachuca, Arizona

ABSTRACT

Project 6X6-01-001-01 Title: Preventive Medicine - Communicable Diseases

Task No. 2 Title: Studies on Penicillin Treatment Failures of Gonorrhea

Reporting Installations: Sixth US Army Medical Laboratory
Fort Baker, California

 US Army Hospital
Fort Huachuca, Arizona

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Adrian D. Mandel, Major, MSC
Edward Kopplin, Jr., Capt, MSC
Robert S. Croissant, Capt, MC
George J. Omi, B.S.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Studies on penicillin treatment failures of gonorrhea were extended and confirmed with the addition of more cases to the study and a change in the treatment schedule. Beginning 1 August 1962, the treatment of gonorrhea was altered from two (2) injections of 1,200,000 units of penicillin (600,000 units of procaine penicillin plus 600,000 units of bicillin), the second dose given 48 hours following the first, to a single dose of 1,200,000 units. Of 75 patients receiving the single dose of penicillin, there were three (3) treatment failures. In each instance of a treatment failure, N. gonorrhoeae, isolated prior to and following treatment, was sensitive to 0.005 units of penicillin/ml. To date, organisms belonging to the Mimae-Herellea group have not been isolated from any of the patients in this study.

Investigations were begun in an attempt to develop a medium for the selective isolation of Mimae-Herellea organisms from urethral discharges and to correlate stained slide readings of urethral discharges with the isolation of Mimae-Herellea organism. It appears that the stained slide diagnosis of gonorrhea by well-trained and experienced individuals is very accurate.

BODY OF REPORT

Project 6X6-01-001-01

Title: Preventive Medicine -
Communicable Diseases

Task No. 2

Title: Studies on Penicillin
Treatment Failures of
Gonorrhea

Description: This is the second annual report of a study designed to:
(1) Isolate and identify by exacting morphological and biochemical studies the etiological agents of gonorrhea at any Army Post experiencing an alleged increase in penicillin treatment failure of gonorrhea. (2) To determine the penicillin resistance of strains of N. gonorrhoeae isolated before and, when present, after treatment. (3) To determine the blood penicillin levels on all patients in an attempt to relate these levels to bacteriological findings. (4) To study the occurrence of Staphylococcus epidermidis and Mimae-Herellea species with N. gonorrhoeae as a possible cause of treatment failure.

Progress: Studies on Penicillin-Resistant Gonorrhea:

Studies on Penicillin-Resistant Gonorrhea.

a. The background of this study and the methods used are described in detail in last year's annual progress report Project 6X6-01-001-01, Preventive Medicine - Communicable Diseases Task 2, Studies on Penicillin Treatment Failures of Gonorrhea, January 1962 through 30 June 1963. The investigation continued with the addition of more cases to the study and, beginning 1 August 1962, a change in the treatment schedule. At that date, the treatment of gonorrhea was altered from (2) injections of 1,200,000 units of penicillin (600,000 units of procaine penicillin plus 600,000 units of bicillin), the second dose given 48 hours following the first, to a single dose of 1,200,000 units. The latter dose is the same dose as was given during the period that the penicillin treatment failures were originally reported.

Due to these reported penicillin treatment failures, the present study was initiated. Therefore, it is of interest to compare the penicillin treatment failure rate of a carefully controlled clinical and laboratory study with the previously reported 30% failure rate which was established without the benefit of laboratory studies. Table I shows the number of treatment failures according to the penicillin dosage used.

TABLE I

PENICILLIN TREATMENT OF GONORRHEA

	<u>2.4X10⁶ unit</u>	<u>1.2X10⁶ 2X 48 hrs. apart</u>	<u>1.2X10⁶</u>	<u>TOTAL</u>
Failure	1	7	3	11
Success	<u>32</u>	<u>58</u>	<u>72</u>	<u>62</u>
TOTAL	33	65	75	173

Failure Rate: 6.5%

b. Of the 173 strains of N. gonorrhoeae isolated and examined for resistance to penicillin, none were resistant to 0.5 units penicillin/ml. one was resistant to 0.3 units/ml, three were resistant to 0.05 units/ml, 19 were resistant to 0.005 units/ml and the remaining 150 strains were sensitive to 0.005 units/ml. The results are tabulated in Table II.

TABLE II

PENICILLIN-RESISTANCE OF N. GONORRHOEA

<u>Units Penicillin G/ml</u>	<u># Isolates</u>
0.5	0
0.3	1
0.1	0
0.05	3
0.005	19
Sensitive to 0.005 Units/ml	150

c. This report, in agreement with the first annual report, notes that in no instance was a treatment failure associated with a resistant strain of N. gonorrhoeae, or development of resistance during treatment. Every case of a treatment failure was caused by an organism resistive to 0.005 units of penicillin/ml. The organisms isolated after treatment were also sensitive to 0.005 units of penicillin/ml.

d. Mimae-Herellea organisms have been reported to be associated with penicillin treatment failures of gonorrhea. However, this point has not been too clearly established and a joint investigation to determine the incidence of Mimae-Herellea organisms in urethral discharges is being performed in cooperation with the U. S. Navy. (Preventive Medicine Unit #5, U. S. Naval Hospital, San Diego, California. Lt. Commander Blasinski is in charge of the Navy effort). At the time that urethral discharges were plated on isolation media, stained smears were made for the purpose of evaluating the stained smear as a diagnostic tool in gonorrhea. The isolation medium used permitted the charac-

teristic growth only of Mimae-Herellea organism, and suppressed the growth of N. gonorrhoeae. To date, 38 patients with a clinical diagnosis of gonorrhea have been examined. Stained smears of each case revealed gram-negative intracellular diplococci, morphologically resembling N. gonorrhoeae. From only one of these patients, was a Mimae-Herellea organism isolated. Although no attempt was made to isolate N. gonorrhoeae it was assumed that they were the etiological agents of gonorrhea in all of these patients.

Summary & Conclusions:

1. A continuation of bacteriological studies of penicillin treatment failures of gonorrhea confirms and extends our earlier findings, that no penicillin-resistant strains of N. gonorrhoeae were associated with treatment failures. In fact, no strains of N. gonorrhoeae resistant to penicillin have been encountered to date. The inclusion of more patients in the study permits a more accurate determination of the treatment failure rate, now established as 6%-10% when 1-2 mega units of penicillin are used. It also appears that administration of penicillin in a single dose, or in equal divided doses, does not effect the failure rate.

2. An organism of the Mimae-Herellea group was isolated only once in this study, and in that instance was not associated with a treatment failure. If it is assumed that gonorrhea caused by "gram-negative intracellular diplococci" is due to "Mimae-Herellea" or N. gonorrhoeae or a mixture of each, and if a culture fails to recover "Mimae-Herellea" (an easy to culture organism) it may be safely assumed that the offending organism is N. gonorrhoeae. On the basis of our attempts to isolate "Mimae-Herellea" in parallel with stained smear studies we therefore conclude that in the hands of well-trained, experienced individuals, the stained smear diagnosis of gonorrhea to be close to 98% accurate.

3. These results are not in agreement with those of Svihus, Lucer, Mikolajczyk and Carter (J.A.M.A., 177: 121, 1961) who reported Mimae as the cause of a gonorrhea-like syndrome resistant to treatment by penicillin. We also do not confirm the findings of Sokoloff and Goldstein (J.A.M.A., 184: 197, 1963) of a correlation of penicillin treatment failures with organisms that were relatively resistant to penicillin. We feel that a possible explanation, and one that is worthy of further study, is the relationship of geographic location to the frequency of organism found as the cause of gonorrhea.

4. In addition to a continuation of the study along present lines, we intend to investigate the incidence of Mimae-Herellea organisms at several geographic locations, and to continue with the development of a medium for the specific, rapid isolation of these organisms.

ANNUAL PROGRESS REPORT

Project 6X61-01-001-01, Communicable Diseases

Task 3, Culture Media for Growth and Maintenance of Tissue Cell Lines

Reporting Installation: Sixth US Army Medical Laboratory
Fort Baker, California

Division of Immunology

Period Covered by Report: 1 July 62 through 30 June 1963

Principal Investigators: Albert Leibovitz, Lt Col, MSC
Charlotte A. John, B.S., M.D.**

Assistants: Raymond P. J. Smith, SSG
Sam B. Ezell, B.S.
Agnes Nakamura, B.A.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project 6X61-01-001-01

Title: Communicable Diseases

Task No. 3

Title: Culture Media for Growth
and Maintenance of Tissue
Cell Lines

Reporting Installation:

Sixth US Army Medical Laboratory
Fort Baker, California

Period Covered by Rep rt:

1 July 1962 through 30 June 1963

Authors:

Albert Leibovitz, Lt Col, MSC
Charlotte A. John, B.S., M.D.
Raymond P. J. Smith, SSG
Sam B. Ezell, B.S.
Agnes Nakamura, B.A.

Reports Control Symbol:

MEDDH-288

Security Classification:

UNCLASSIFIED

Approximately 140 modifications of Medium L-15 have been studied in the past year in an attempt to obtain a synthetic medium for the rapid growth of normal and malignant tissue cell cultures without success. Several chemicals appear promising and further studies are indicated. Medium L-15 has proved highly successful in the growth of all normal and malignant cell cultures studied when fortified with serum. Being glucose and bicarbonate free, ready growth was obtained in free gas exchange with the atmosphere thereby eliminating one of the serious drawbacks in routine tissue culture studies. A manuscript on this medium has been accepted for publication by the American Journal of Hygiene.

BODY OF REPORT

Project No. 6X61-01-001-01

Title: Communicable Diseases

Task No. 3

Title: Culture Media for Growth
and Maintenance of Tissue
Cell Lines

Description: The purpose of the present investigations is to develop an all-purpose medium, preferably completely synthetic, that will enable the rapid growth and maintenance of both normal and malignant cell cultures and will enhance the growth of virus agents therein.

Progress:

1. The necessity of body fluids, especially serum, for the growth of most tissue cell cultures leads to peaks and valleys in tissue cell growth. Serum optimal for cell growth leads to luxuriant growth of all cell cultures studies with medium L-15. However, the reaction of the cells to various lots from the same type host, i.e., foetal calf serum, may differ markedly and even lead to the death of the cell strain. The "toxic" effect may be insidious and the cell strain badly hurt before these effects are obvious. Puck et al (J. Exp. Med. 108: 945, 1958) advocate testing by the use of cell clones. However, most laboratories are conveniently located near their source of such products and this difficulty can only be overcome by stock-piling large amounts of non-toxic sera of proven value.

2. In an attempt to eliminate serum from the medium for cell propagation, approximately 140 media have been composed to date. As yet, none have approached the serum containing medium for cell growth beyond the second or third subculture. Certain products show definite promise however, and will be further investigated. These include protamine, protamine zinc insulin, methocel, the alpha globulin fractions of various sera, ovomucoid, and some of the enzymatic hydrolytic products of various proteins. Although the aforementioned products are not truly "synthetic", they can be more readily standardized than serum and usable lots can be studied as to their chemical make-up.

3. Medium L-15, utilizing 10 per cent foetal calf serum for cell propagation and 2 per cent for cell maintenance, has proved highly successful for the growth of both normal and malignant cell lines. As this medium permits the growth of all cell strains in free gas exchange with the atmosphere and enables virus growth to at least the same extent as in glucose-bicarbonate mediums, a manuscript was prepared for publication which has been accepted by the American Journal of Hygiene for possible September publication. This manuscript is hereby duplicated, as it covers the use of this medium in tissue cell growth and maintenance.

Morgan (1) in his review of tissue culture nutrition cites the need of a non-bicarbonate buffer to simplify culture techniques. For the past two years, such a medium (L-15) has been in use for the growth and maintenance of tissue cell cultures in a diagnostic virus laboratory. The use of the free base amino acids, especially L-arginine, and the substitution of D (+) galactose, sodium pyruvate and DL-alpha alanine for glucose has yielded the desired medium without any loss in the rate of tissue cell proliferation or in the ability to isolate and propagate virus agents in cell lines normally attained with glucose-containing mediums.

MATERIALS AND METHODS

Medium L-15: Preparation of the necessary solutions and the formula for medium L-15 are presented in tables 1 and 2. The final pH, without the addition of any exogenous buffer, is 7.6. The free base amino acids of L-arginine, L-histidine and L-cysteine are used. L-glutamine, because of its instability, is not added to the final medium until just before use. Foetal calf serum is added in desired concentrations; 10 per cent is normally used for the growth of all cultures, 2 per cent for maintenance.

Medium L-15G: Similar to L-15, but glucose in place of galactose.

Vitamins: Same concentration employed as recommended by Eagle (2). See table 1.

Salt solution: Modified Hank's BSS (3) with double concentration of the magnesium salts after the findings of Waymouth (4).

Tissue cell lines: The malignant cell lines, HEp-2¹ and stable monkey kidney (SMK)² were used throughout this study. Each has been passed over 100 times in medium L-15. Various primary cell cultures have been initiated from their original tissues and continued in serial passage as diploid cell strains³. For twice-weekly harvests, 1,000,000 cells in 20 ml of medium were inoculated in 16-ounce prescription bottles⁴; for weekly harvests, 500,000 cells. Primary cell strains required a minimum inoculation of 2,000,000 cells in 20 ml of medium and were harvested twice weekly (5). Both malignant and normal cell cultures had a complete change of medium on the following day. This sufficed when harvesting on a twice-weekly basis; otherwise, they were fed 3 times a week. Tubes were prepared from malignant cell lines by inoculating 20,000 cells in $\frac{1}{2}$ ml of medium. The medium was completely changed on the following day and 1 ml

1. Microbiological Associates, Albany, California
2. Kindly furnished by Dr. Robert N. Hull, Eli Lilly and Co., Strain LLC-Mk₂ (NCTC-3196).
3. Dr. Charlotte John originated most of these cell lines and has maintained them in serial passage. These studies are to be published.
4. Demuth or Brockway.

TABLE 1
Ingredients of Medium^a

<u>Solution 1: Amino acids^b</u>		<u>Solution 2: Vitamins^c</u>		<u>Solution 6: Galactose^c</u>	
<u>Amino acid</u>	<u>mg/L</u>	<u>Vitamin</u>	<u>mg/L</u>	<u>D (+) Galactose</u>	<u>mg/L</u>
DL- α alanine	450	Choline	1.0	<u>Solution 7: Salts^e</u>	900
L-Arginine (free base)	500	Folic acid	1.0	NaCl	8000
L-asparagine	250	Inositol	2.0	KCl	400
L-Cysteine (free base)	120	Nicotinamide	1.0	MgSO ₄ ·7H ₂ O	200
Glycine	200	DL-Ca Panthothenate	1.0	MgCl ₂ ·6H ₂ O	200
L-histidine (free base)	250	Pyroxidine HCl	1.0	<u>Solution 8: salts^e</u>	
DL-Isoleucine	250	Riboflavin-5-phosphate, Na	0.1	CaCl ₂	140
L-leucine	125	Thiamin monophosphate	1.0	<u>Solution 9: Salts^e</u>	
L-lysine	75	Na ₂ HP0 ₄	140.00	Na ₂ HP0 ₄	50
DL-methionine	150	<u>Solution 3: L-Glutamine^c</u>		KH ₂ PO ₄	60
DL-phenylalanine	250	L-Glutamine	300.00	Phenol Red ^f	10
L-serine	200	<u>Solution 4: L-Tyrosine^d</u>		<u>Solution 10: Antibiotics^g</u>	
DL-threonine	600	L-tyrosine	300.00	Penicillin	300 μ /ml
L-tryptophane	20	<u>Solution 5: Na Pyruvate^c</u>		Streptomycin	300 mcg/ml
DL-valine	200	Na Pyruvate	550.00	Fungazone	3 μ g/ml

- a. All chemicals purchased from Nutritional Biochemical Corp. with exception of phenol red and antibiotics.
- b. Prepared as 10X, millipore filter sterilized, dispensed in 100 ml amounts, stored at 4°C.
- c. Prepared as 100X, millipore filter sterilized, dispensed in 10 ml amounts, stored at -20°C.
- d. Prepared as 1X, dispensed 750 ml/1,000 ml flask, autoclaved 121°C/10 minutes, stored room temperature.
- e. Solutions 7, 8 and 9 are prepared as 10X. Solution 7 is prepared in 500 ml distilled water; solution 8 in 100 ml distilled water and solution 9 in 275 ml distilled water plus 25 ml of 0.4 per cent phenol red solution (see note "f"). Each is autoclaved separately, cooled to at least room temperature (preferably overnight in the refrigerator) and then solution 8 is added to solution 7 and finally solution 9 is added to the 7-8 combination. The media is dispensed aseptically in 100 ml amounts and stored at room or refrigerator temperature.
- f. Prepare 0.4 per cent phenol red by placing 1 gram in a 500 ml Erlenmeyer flask. Slowly add N/20 NaOH until the phenol red is almost in solution. This will require about 60 ml. Add additional N/20 NaOH dropwise until all of the phenol red is in solution. Color should be deep red and not purple. QS to 250 ml with distilled water.
- g. All antibiotics combined and prepared as 100X with sterile distilled water, distributed 10 ml amounts, stored at -20°C.

TABLE 2
Formula for medium per liter.^a

Solution	Volume	Distribution and Use
Tyrosine (1X)	750.00 ml	Dispense aseptically in 99 ml
Vitamins (100X)	10.00	amounts. Just before use add
Pyruvate (100X)	10.00	1 ml L-Glutamine (100X) per
Galactose (100X)	10.00	bottle. Foetal calf serum
Salts (10X))	100.00	added as desired, usually 10
Amino acids (10X)	100.00	per cent for growth and 2 per
Antibiotics (100X)	10.00	cent for maintenance.

a. Final pH of medium, without addition of exogenous buffers, will be about 7.6.

of fresh growth medium was added per tube. Cells were ready for virus diagnostic work by the third day; the growth medium was replaced with maintenance medium. Primary cell strains were similarly prepared except that 50,000 cells per $\frac{1}{2}$ ml of medium were inoculated per tube.

Free atmosphere exchange: All cell cultures were grown in free gas exchange with the atmosphere. Tubes were prepared in the wash room by setting up 16 x 125 tubes in Thomas tissue culture racks² with the diamonds facing the wire springs. A heavy brown paper cap was made to fit the entire rack and was taped on about $\frac{1}{2}$ -inch above the tubes and the entire unit autoclaved at 121C for 15 minutes. In the tissue culture propagation room, the paper cap was removed, $\frac{1}{2}$ ml of cells inoculated per tube using an automatic syringe³, the top of the tubes were flamed and the paper cap replaced. The rack was slanted at 5 degrees in a 35C incubator. The following day the media was changed by decanting the entire rack, flame sterilizing the tube mouths, and inoculating 1 ml of fresh medium into each tube. The rack was recapped with the paper shield and incubated. When tubes were ready for the viral diagnostic room, they were fed with maintenance medium and capped with sterile, rubber-lined, screw caps.

Harvesting of cell lines: A modification of the techniques of Gwatkin and Siminovich (6) using 0.05 per cent versene¹ and 0.05 per cent trypsin in Hank's balanced salt solution without calcium or magnesium proved very successful for both normal and malignant cell cultures. The final pH of the medium was 7.6 without the addition of any exogenous buffer. The growth medium was removed from the tissue cell bottle and replaced with 10 ml of the enzyme solution. The bottle was rotated by hand about 20 times and placed in the 35C incubator for 5 minutes. The cell suspension was gently pipetted and used to wash the glass wall to remove any cells still adhering and then transferred to a 50 ml screw-capped centrifuge tube. Centrifuged at 600 rpm for 5 minutes. The supernatant was removed and discarded. Ten ml of complete growth media were added and the cells gently pipetted into suspension. The cells were counted, using a hemacytometer, and diluted with complete medium to the desired concentration for fresh bottles or tubes.

Prototype virus agents: Almost all prototype viruses had been obtained by predecessors of the author at this laboratory. They were received from the Walter Reed Army Institute of Research, The National Institute of Health and from the California State Department of Health. The Sabin attenuated poliovirus agents, types 1, 2, and 3 were purchased as commercial vaccine⁴.

-
1. Arthur Thomas Co., Philadelphia, Pennsylvania.
 2. Becton, Dickinson and Co., Rutherford, New Jersey.
 3. Nutritional Biochemical, Cleveland, Ohio.
 4. Pfizer Laboratories, New York, New York.

RESULTS

Growth and maintenance of cell lines: Freshly harvested cells sub-cultured in medium L-15 containing 10 per cent foetal calf serum required from 2 to 4 hours to become attached and flattened on the bottle wall. Although the cells spread soon after attachment, there was a lag phase of approximately 24 hours before active multiplication was entered, the malignant cell lines required about 18 hours per generation. The HEP-2 and SMK lines usually increased 8-fold in 3 to 4 days and 12- to 20-fold in 7 days. The primary cell strains, when retained in the rapid growth phase, behaved similarly. However, these fibroblast-like cells were several times larger than the epithelial malignant cells and their growth was more rapidly restricted by the size of the bottle employed. Normally a 3-fold increase in numbers filled the bottle. The pH of the medium remained constant throughout the growth cycle without the addition of exogenous buffers.

Medium L-15G, incorporating glucose instead of galactose, showed a similar growth pattern with HEP-2 cells. Studies were not made with the SMK or primary cell cultures. Although the cells grew satisfactorily in the free gas exchange with the atmosphere, within 48 hours after inoculation the medium had become markedly acid. Changing of the medium would only maintain the pH on the alkaline side for short periods of time, e.g., 1-2 hours. Once sodium bicarbonate was added to help maintain the pH, the bottles had to be tightly capped and the medium would again be markedly acid by the following morning.

Eagle's MEM (2) was purchased as a complete medium¹. Freshly harvested HEP-2 cells appeared to grow in this medium but could not overcome the initial sharp rise in pH even though the bottles were tightly capped and within 72 hours the cells started to degenerate. Cells established on L-15 could be fed with Eagle's MEM without any obvious untoward reaction. The TCID₅₀ experiments described below, utilizing Eagle's MEM, were accomplished with tubes of HEP-2 cells established on L-15 medium.

Isolation of virus agents from specimens: The majority of specimens were from Armed Forces personnel in the 18 to 40 year age bracket. Virus agents isolated during this study period in the HEP-2 and SMK cell lines included poliovirus types 1, 2, and 3; Coxsackie Group A, type 9; Coxsackie Group B, types 1 through 5; ECHO types 2, 4, 6, 8, 9 and 11; adenovirus types 1, 2, 3, 4, 5, 7, and 10; herpesvirus, parainfluenza type 3; and several unidentified agents. Primary cell strains, especially embryonic lung cells, yielded many of the above, plus several unidentified agents.

Growth of prototype viruses: Prototype virus agents of poliovirus types

1. Microbiological Associates.

1, 2, and 3; Coxsackie, Group A, types 7, 9, 10, 11, 13, 14, 15, 17, 18, and 21; Coxsackie, Group B, types 1 through 6; ECHO types 1 through 22; Adenovirus types 1 through 10; herpesvirus and parainfluenza type 1 were adapted to HEp-2 and SMK cells in medium L-15. Influenza A and B strains failed to grow beyond the second passage as indicated by the hemadsorption test. Studies with primary cell lines are still in the pilot stage and will be included in a later report.

The 50 per cent tissue culture infective dose (TCID₅₀) was calculated by the method of Reed and Muench (7) in comparing the growth of various viral prototype agents in medium L-15, bicarbonate free, in both free gas exchange with the atmosphere and in tight-capped tubes, and in Eagle's MEM (0.22 per cent sodium bicarbonate) in tightly-capped tubes. It soon became apparent that the "optimal" medium varied with the prototype virus under study. See table 3. Five tubes of HEp-2 cells, in 66th passage in L-15, 4 days of age were used for each dilution and the TCID₅₀ was determined 7 days after inoculation of the virus agents. The attenuated poliovirus Sabin strains employed in commercial vaccine showed the monovalent types 1 and 3 to grow best in L-15 in free gas exchange with the atmosphere, type 2 gave the highest titer in MEM. The virulent strain of poliovirus type 1, Mahoney, also grew best in MEM, but ECHO type 2, Cornelius strain, preferred L-15 in either loose or tight-capped tubes.

DISCUSSION

Development of medium L-15 was made in an effort to obtain a medium that would enhance both tissue cell and virus growth. Approximately 140 media have been composed to date in the study of various compounds in tissue cell growth and maintenance, but none of the variations have demonstrated any significant increased benefit. The basic studies of Eagle and his co-workers (2, 8-15) were chosen as the model for these studies as theirs was the simplest (approaching a synthetic medium) composed to date that would enable the growth of most tissue cell cultures. Evaluation of their data for the growth of human (10), mouse (9) and monkey kidney cells (13) revealed that the minimal amounts of each amino acid were employed which would yield optimal growth of the cell line under study. Many of these amino acids are rapidly depleted during cell growth (16). The finding of Dubes (17) that there may actually be competition between cell and virus for certain essential amino acids led to the thesis of incorporating into the growth medium the maximum amount of each amino acid which might be employed without seriously retarding cell growth. The first of such media to yield rapid cell growth was L-9¹. See table 4. Asparagine (18), glycine (13, 19) and serine (15, 20, 21) were added as these amino acids were essential for certain cell lines or stimulated growth. Although L-9 promoted rapid growth of HEp-2 cells² and Hela cells²

1. This medium was devised while at the Fifth US Army Medical Laboratory in 1959.

2. Microbiological Associates.

TABLE 3

Comparative TCID₅₀ titers of prototype viruses under varying atmosphere, carbohydrate and bicarbonate factors.^a

VIRUS	TYPE	Medium L-15 Loose Caps ^b	Medium L-15 Tight Caps ^c	Eagle's MEM (2) Tight Caps ^d
Poliovirus Sabin Vaccine ^e Lot #103113713	1	6.6 ^g	6.5	6.2
Poliovirus Sabin Vaccine ^e Lot #1031125-4	2	6.2	6.5	7.5
Poliovirus Sabin Vaccine ^e Lot #2031149-4	3	7.2	6.6	6.6
Poliovirus Mahoney ^f N646	1	6.6	6.5	6.8
ECHO Cornelius ^f TC-2661	2	7.2	7.2	6.5

a. Five tubes HEP-II cells per dilution, 66th passage in medium L-15, 4 days old. TCID₅₀ determined 7 days after inoculation of virus.

b. Free gas exchange with atmosphere, no bicarbonate in medium.

c. No free exchange with atmosphere, no bicarbonate.

d. No free exchange with atmosphere, 0.22 per cent bicarbonate.

Purchased as complete medium from Microbiological Associates.

e. Pfizer Laboratories, New York City, New York.

f. Obtained from Walter Reed Army Institute of Research.

g. TCID₅₀ per 0.1 ml log₁₀.

(from 8- to 16-fold increase in 7 days), this medium had the same disadvantages as other glucose and bicarbonate containing media. The rapidly growing cells would reduce the pH of the medium in direct proportion to the cell population. By the time tubes were 7 to 10 days of age, the pH would often drop from 7.6 to 6.0 overnight.

Chang and Geyer (22) noted that d(+) galactose in the presence of pyruvate and alpha alanine could be consistently substituted for glucose in the growth of HeLa and human conjunctival cells. Eagle, et al, (14) confirmed these findings and also noted, in contrast to glucose, only a small portion of the galactose utilized was converted to lactic acid even though cell growth was not diminished. Without pyruvate, growth was haphazard. Coupling this information with the knowledge that much larger amounts of amino acids could be employed than were originally recommended by Eagle, an effort was made to eliminate the unstable bicarbonates as a buffering system by utilizing commercially available free base amino acids. At present, arginine, histidine and cysteine are available and were incorporated. L-arginine, free base, was chosen for pH adjustment of the medium. About 3uM/ml yielded the desired pH 7.6 thereby negating the necessity for any other exogenous buffer. This concentration of arginine approached the maximum amount usable (9, 10) (see table 4); others (23, 24) have noted that similar amounts could be employed without toxic manifestations. Although Thomas, et al (24) found that 42 mcg/ml arginine yielded maximum stimulation of growth of various cell lines in suspension, additions of 336 mcg/ml had no toxic effect. Piez and Eagle (25) noted that arginine was rapidly depleted during cell growth and was not concentrated in the free amino acid pool.

Medium L-15 promoted the growth of HEp-2, stable MK cells, and a variety of primary human cell strains, both embryonic and adult, to at least the same extent as the glucose containing media. Up to 20-fold increase in cell population was obtained with the malignant cell lines in 7 days. The growing of cells in free exchange with the atmosphere enabled the medium to remain at a constant pH throughout the growth cycle. The excellent growth in medium L-15 is partially attributed to the elimination of rapid fluctuations in pH with its attending toxicity (26).

Although several investigators (27, 28, 29) have demonstrated the need of exogenous bicarbonate for optimal virus growth in glucose media, studies in virus titrations with prototype virus strains indicate that such is not essential in medium L-15 (see table 3). Eagle (8) has noted that exogenous bicarbonate is not necessary for the growth of HeLa cells. The possibility of growing cells in free atmosphere exchange allows the preparation and handling of large numbers of bottles and tubes by one technician. The use of a sterile paper cap for the entire rack of tubes is sufficient to prevent overt contamination.

The high levels of amino acids employed in medium L-15 are not essential to cell growth; growth can be obtained using one-tenth the concentration. The amounts are empirical with the hope of obtaining virus

TABLE 4
Evaluation of Eagle's Amino Acid Data^a

AMINO ACID	EAGLE 1955(10) $\mu\text{M}/\text{ml}$	EAGLE 1959(2) $\mu\text{M}/\text{ml}$	Minimum to Maximum range Evaluation	Amount used Medium L-15 $\mu\text{M}/\text{ml}$ ^b
L-Arginine	0.10	0.60	0.10 to 3.00	3.00
L-Cystine	0.05	0.10	0.01 - 0.10	NA ^c
L-Histidine	0.02	0.20	0.03 - 10.00 ^d	1.50
L-Isoleucine	0.20	0.40	0.10 - 0.30 ^e	1.00
L-Leucine	0.10	0.40	0.10 - 1.00	1.00
L-Lysine	0.10	0.40	0.10 - 1.00	0.50
L-Methionine	0.05	0.10	0.03 - 1.00	0.50
L-Phenylalanine	0.05	0.20	0.03 - 1.00	0.75
L-Threonine	0.10	0.40	0.10 - 3.00	2.50
L-Tryptophan	0.01	0.05	0.03 - 0.10	0.10
L-Tyrosine	0.10	0.20	0.03 - 3.00	2.00
L-Valine	0.10	0.40	0.10 - 1.00	1.00

- a. Personal evaluation of Eagle's data for growth of Hela cells(10).
b. Medium L-9 same as above except that 1.5 $\mu\text{M}/\text{ml}$ L-Arginine HCL used in place of the 3 $\mu\text{M}/\text{ml}$ L-arginine free base in L-15. Where the DL compounds are employed, twice the above concentrations are used.
c. L-cysteine used 1.00 $\mu\text{M}/\text{ml}$ after data of Morgan (32).
d. Double peak (?) at 1.0 $\mu\text{M}/\text{ml}$ and at 10 $\mu\text{M}/\text{ml}$.
e. Maximum growth at 0.3 $\mu\text{M}/\text{ml}$, but only gradual drop in growth at higher levels.

growth from disease entities in which no agent has been isolated to date. A variety of primary cell strains are under study to obtain strains susceptible to a wide virus spectrum. Medium L-15 coupled with the HEp-2 cell line allows excellent growth of the adenovirus and herpesvirus. Excellent complement fixation antigen are prepared from both agents using the basic methods of Schmidt, Lennette, and Shon (30).

Cooper (31) found the efficiency of viral plaque formation to be superior when galactose was substituted for glucose. Although he does not reveal how his medium is buffered, he was able to eliminate the carbon dioxide gassing incubator.

SUMMARY

A medium (L-15) has been devised that permits the growth and maintenance of normal and malignant tissue cell cultures in free gas exchange with the atmosphere.

Free base amino acids, especially L-arginine, are substituted for bicarbonate as the buffer. D (+) galactose, sodium pyruvate, and DL-alpha alanine are substituted for glucose. The maximum amount of each of the essential amino acids is employed that does not retard cell growth.

This medium permits the growth of various virus agents to at least the same extent as bicarbonate glucose media.

REFERENCES

1. Morgan, J. F. Tissue culture nutrition. *Bact. Rev.*, 1958, 22: 20-45
2. Eagle, H. Amino acid metabolism in mammalian cell cultures. *Science*, 1959, 130: 432-437.
3. Paul, J. Cell and tissue culture, 2d Ed., Williams and Wilkins Co., Baltimore, 1960, p. 81.
4. Waymouth, C. Rapid proliferation of sublines of NCTC Clone 929 (Strain L) mouse cells in a simple chemically defined medium (MB 752/1). *Jour. Nat. Cancer Inst.*, 1959, 22: 1003-1017.
5. Hayflick, L. and Moorhead, P. S. The serial cultivation of human diploid cell strains. *Exper. Cell Research*, 1961, 25: 585-621.
6. Gwatkin, R. B. L. and Siminovitch, L. Multiplication of single mammalian cells in a non-carbonate medium. *Proc. Soc. Exper. Biol. and Med.*, 1960, 103: 718-721.
7. Reed, L. J. and Muench, H. A. A simple method of estimating fifty per cent endpoints. *Amer. Jour. Hyg.*, 1938, 27: 493-497.
8. Eagle, H. The salt requirements of mammalian cells in tissue culture. *Arch. Biochem. Biophys.*, 1956, 61: 356-366.
9. Eagle, H. The specific amino acid requirements of a mammalian cell (Strain L) in tissue culture. *Jour. Biol. Chem.*, 1955, 214: 839-853.
10. Eagle, H. The specific amino acid requirements of a human carcinoma cell (Strain HeLa) in tissue culture. *Jour. Exper. Med.*, 1955, 102: 37-48.
11. Eagle, H. The minimum vitamin requirements of the L and HeLa cells in tissue culture, the production of specific vitamin deficiencies, and their cure. *Jour. Exper. Med.*, 1955, 102: 595-600.
12. Eagle, H., Oyama, V. I., Levy, M. and Freeman, A. E. myo-inositol as an essential growth factor for normal and malignant human cells in tissue culture. *Jour. Biol. Chem.*, 1957, 226: 191-207.
13. Eagle, H., Freeman, A. E. and Levy, M. The amino acid requirements of monkey kidney cells in first culture passage. *Jour. Exper. Med.*, 1958, 107: 643-652.
14. Eagle, H., Barban, S., Levy, M., and Schulze, H. O. The utilization of carbohydrates by human cell cultures. *Jour. Biol. Chem.*, 1958, 233: 551-558.

15. Eagle, H. and Piez, K. The population-dependent requirement by cultured mammalian cells for metabolites which they can synthesize. *Jour. Exper. Med.*, 1962, 116: 29-43.
16. Westfall, B. B., Peppers, E. V., and Earle, W. R. The change in concentration of certain constituents of the medium during growth of the strain Hela cells. *Amer. Jour. Hyg.*, 1955, 61: 326-333.
17. Dubes, G. R. Cystine requirements for normal poliovirus action on monkey kidney tissue cultures. *Proc. Soc. Exper. Biol. and Med.*, 1956, 93: 129-132.
18. Neuman, R. E. and McCoy, T. A. Dual requirement of Walker carcinosarcoma 256 in vitro for Asparagine and Glutamine. *Science*, 1956, 124: 124-125.
19. Melnick, J. L., Hsuing, G. D., Rappaport, C., Howes, D., Reissig, M. Factors influencing the proliferation of viruses. *Texas Rep. Biol. and Med.*, 1957, 15: 496-533.
20. Haff, R. F. and Swim, H. E. Amino acid requirements of rabbit fibroblasts, Strain RM3, *Fed. Proc.*, 1956, 15: 591.
21. Lockart, R. Z., Jr., and Eagle, H. Requirements for growth of single human cells. *Science*, 1959, 129: 252-254.
22. Chang, R. S. and Geyer, R. P. Propagation of conjunctival and Hela cells in various carbohydrate media. *Proc. Soc. Exper. Biol. and Med.*, 1958, 96: 336-340.
23. Morgan, J. F., Morton, H. I., and Pasieka, A. E. The Arginine requirement of tissue cultures. I. Interrelationships between Arginine and related compounds. *Jour. Biol. Chem.*, 1958, 233: 664-666.
24. Thomas, W. J., Ziegler, D. W., Scheparts, S. A., and McLimans, W. F. Use of Arginine to eliminate medium changes in tissue culture systems. *Science*, 1958, 127: 591-592.
25. Piez, K. A., and Eagle, H. The free amino acid pool of cultured human cells. *Jour. Biol. Chem.*, 1958, 231: 533-545.
26. Mackenzie, C. G., Mackenzie, J. B., and Beck, P. The effect of pH on growth, protein synthesis, and Lipid-Rich particles of cultured mammalian cells. *Jour. Biophys. and Biochem. Cytol.*, 1961, 9: 141-156.
27. Sabin, A. B. Properties of attenuated polioviruses and their behavior in human beings. *Spec. Publ. N. Y. Acad. Sci.*, 1957, Part 1, 5: 113-140.

28. Chang, R. S. Participation of bicarbonate in RNA and protein synthesis as indicated by virus propagation in human cells. Jour. Exper. Med., 1959, 109: 229-238.
29. Mosley, J. W. and Enders, J. F. Growth of poliovirus in cotton-plugged monkey kidney cell cultures. Virology, 1962, 17: 251:261.
30. Schmidt, N. J., Lennette, E. H., and Shon, C. W. A complement-fixing antigen for Herpes simplex derived from chick embryo tissue cultures. Amer. Jour. Hyg., 1960, 72: 59-72.
31. Cooper, P. D. An improved agar cell-suspension plaque assay for poliovirus: some factors affecting efficiency of plating. Virology, 1961, 13: 153-157.
32. Morgan, J. F. and Morton, H. J. Studies on the sulphur metabolism of tissues cultivated in vitro. III. Interrelationships between cysteine, methionine, cysteamine, and cystamine. Canad. Jour. Biochem. and Physiol., 1957, 35: 785-794.